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A combined FTIR and DSC study on the bilayer-stabilising effect of electrostatic interactions in ion paired lipids

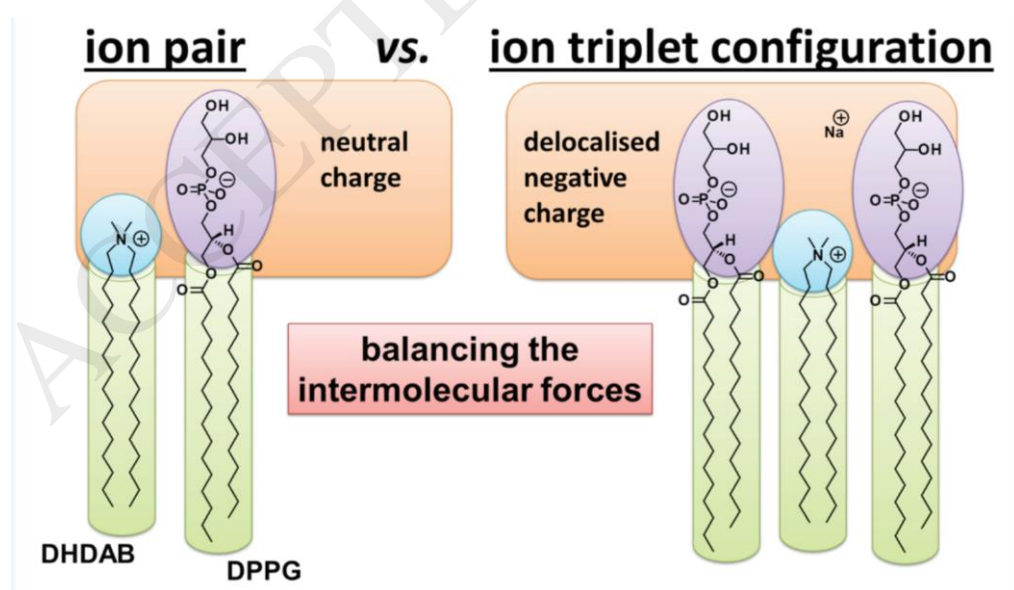
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Graphical Abstract



Highlights

- DPPG and DHDAB form an ion pair which acts as cluster compound.
- The equimolar DPPG/DHDAB mixture is characterized by a neutralization of the charge.
- The DPPG/DHDAB 2/1 mixture is characterized by the formation of gel phase stabilising ion triplets.

Abstract

Investigating lipid ion pair formation is important for understanding the mechanisms of lipid-mediated drug resistance in bacteria. In this study we have used the charged amphiphiles dipalmitoylphosphatidylglycerol (DPPG) and dihexadecyldimethylammonium bromide (DHDAB), as a model to evaluate the formation of ion pairs by a combined Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) analysis. FTIR was employed to study the environment of the DPPC headgroup phosphate and lipid/surfactant alkane chains, in vesicles formed by the two amphiphiles mixed in various molar ratios. An increase of the absorbance ratio of 1221 to 1201 cm^{-1} in the asymmetric phosphate stretching mode was found to follow a sigmoidal relationship with the proportion of DHDAB, increasing to a plateau above a DPPG/DHDAB 1:1 molar ratio of, providing evidence that the PG headgroup phosphate is involved in ion pairing. A consistent red shift was measured for the position of the symmetric CH_2 stretch band for the lipid/surfactant 1:1 molar ratio mixture, which is indicative of an increased ordering of the hydrophobic chains. The DSC experiments yielded information about the thermotropic and the mixing behaviour of the lipid/surfactant systems. DPPG and DHDAB seem to form an ion pair with cluster compound characteristics at the equimolar ratio. Most interestingly, the DPPG/DHDAB 2:1 molar ratio mixture is characterized by strong intermolecular interactions, which result in a pronounced stabilization of the gel phase, possibly through the formation of a closely-associated ion triplet configuration in which the charges are delocalised across the headgroups.

Keywords

Model bacterial membranes, differential scanning calorimetry, Fourier transform infrared spectroscopy, ion pair, zeta potential, liposome

1. Introduction

The vast diversity of lipid species present in biological membranes and their compositional plasticity are considered to be important for maintaining the functional homeostasis of membrane proteins in response to environmental fluctuations in temperature, pH and solute content.[1] Alterations in the lipid composition of some biomembranes may also induce changes in membrane function, facilitated solely by lipid-lipid interactions. Such lipid-mediated functional plasticity plays an important role in the survival mechanisms of bacteria exposed to environmental stress.[2] In the case of the Gram positive opportunistic pathogen *Staphylococcus aureus*, increased synthesis of the aminoacyl lipid lysyl-phosphatidylglycerol (L-PG), has been shown to protect the bacteria from defensive cationic membrane-active antimicrobial peptides (AMPs) and thus allow them colonise human epithelia and even establish, maintain and spread infections[3]. The formation of ion pairs between the phosphate of the phosphatidylcholine (PG) headgroup and the amines of the L-PG headgroup, has been shown to fine tune both the charge and ordering of lipids in reconstituted *S. aureus* lipid membranes, thus attenuating interaction with AMPs.[4] The ability of L-PG and other aminoacyl lipids to protect bacteria from host innate immunity has usually been studied in simple model systems, designed to examine the effect of lipid composition on AMP binding, rather than focusing on how L-PG influences the physical properties of lipid membranes.[5] The use of synthetic L-PG for examining its behaviour and influence in model membranes is complicated by its alkali-lability[6] and multiple headgroup pK_a [4], which also exist in synthetic L-PG analogues.[7, 8] In this study we have employed a very simple model system

with which to explore the physicochemical effects of lipid ion pairing on membrane properties, using a range of different techniques.

We chose a structurally simple binary lipid system to evaluate different methods used for the investigation of ion pair formation, using various mixtures of the anionic phospholipid dipalmitoylphosphatidylglycerol (DPPG) and the quaternary ammonium surfactant dihexadecyldimethylammonium bromide (DHDAB) (Figure 1). The combination of techniques we have used has allowed us to differentiate between simple charge neutralization between the anionic DPPG and cationic DHDAB and the formation of real ion pairs, which act as a complex compound. Although our model is structurally very different from the natural PG/L-PG mixture, it possesses various desired characteristics needed to prove the utility of the investigation methods. With respect to the design of our Fourier transform infrared spectroscopy (FTIR) experiments, it was necessary to avoid the presence of phosphate and carbonyl functions in both lipids, because the IR bands of both groups are very sensitive to lipid packing and hydration characteristics. Using DHDAB has several advantages, its lack of both phosphate and ester groups prevents masking of the PO_2^- stretch bands for the DPPG, and its two C16 alkyl chains reduce the risk of packing inhomogeneity arising from hydrophobic mismatch. The various possible intermolecular interactions within DPPG/DHDAB mixtures, are summarised in Figure 1.

The effect of ion pairing on the surface charge properties and dispersion stability of DPPG/DHDAB vesicles in the gel phase was determined using a combination of zeta potential and dynamic light scattering (DLS) measurements. FTIR spectra provided a useful tool for quantifying DPPG/DHDAB ion pairs through analysis of the phosphate stretch bands,[9] and lipid ordering effects were determined by analysing the CH_2 stretching modes.[10] Differential scanning calorimetry (DSC) experiments were performed to get information about the thermotropic behaviour of the lipid mixtures, and therefore allowed an

assessment of their mixing behaviour, and the effect of ion pairing on gel phase stability. This combination of techniques and the results obtained in the DPPG/DHDAB system, sheds light on the complexity of the mixing behaviour of the two lipids and demonstrates that neutralisation of the lipid dispersions does not yield the most stable bilayer structures. This has a number of implications for both the stability of bacterial membranes containing ion paired lipids and their interactions with components of the innate immune system.

2. Materials and Methods

2.1 Materials

High Purity Water with a specific resistivity of 18.2 M Ω .cm and a pH between 5 and 6, was used throughout these experiments (ElgaPURELAB[®]Ultra, Elga Veolia, High Wycombe UK). 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-(*rac*-1-glycerol) sodium salt (DPPG) was supplied by Avanti Polar Lipids (Alabaster, AL, USA) and dihexadecyldimethylammonium bromide with a purity of 97% was purchased from Sigma Aldrich (Poole, Dorset, UK). Analytical grade chloroform and Methanol were purchased from either Fisher Scientific (Loughborough, UK), or Merck (Darmstadt, Germany).

2.2 Zeta-potential and particle size measurements

Lipid/surfactant dispersions were prepared by the film hydration procedure to obtain multilamellar vesicles,[12] from DPPG/DHDAB mixtures dissolved in chloroform/methanol (9/1, v/v). Lipid films of different mixtures ($x_{\text{DHDAB}} = 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, \text{ and } 0.8$) and of the pure lipids were obtained by evaporating the solvent at 40°C for 1 h at 200 mbar and for a further 3 h at 10 mbar. After formation of dry lipid films, sterile filtered dispersion medium (1 mM NaCl to get a suitable conductivity for the ζ -potential measurement), was added to give a final total lipid/surfactant concentration of 0.025 mg mL⁻¹. The lipid dispersions were then incubated at 60°C (at least 5°C above the gel–fluid phase transition of

the phospholipids) while shaking (1400 rpm) for 30 min (Eppendorf Thermomixer 5436). Finally, the lipid dispersions were sonicated at 37 kHz (100% power output, sonication bath FB11201, Fisherbrand, FisherScientific, Germany) for 6 min.

The electrophoretic measurements were performed using the laser-Doppler-electrophoresis technique on a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Malvern, UK) at 25°C. Three measurements consisting of 30 runs with a voltage of 50 V were performed. For the calculations, a viscosity of $\eta=0.8872$ mPa s, a dielectric constant of $\epsilon=78.5$ F m⁻¹, and a refractive index of 1.33 were assumed. The analysis was performed using the Zetasizer software (version 6.34). The mobility μ of the diffusing aggregates was converted into the ζ -potential using the Smoluchowski relationship $\zeta=\mu\eta/\epsilon$. DLS measurements were also performed with the Zetasizer Nano ZS ZEN3600. The scattering angle was 173°. Three measurements consisting of 15 runs with a duration time of 20 s for each run were performed at 25°C. For the calculations, a viscosity of $\eta = 0.8872$ mPa·s and a refractive index of 1.33 were assumed. The Zetasizer software (version 6.34) and the ALV-Correlation software version 3.0 calculated the z-average (diameter) and PDI using the cumulants method, from the intensity distribution autocorrelation function.

2.3 FTIR measurements

DPPG/DHDAB mixtures with DHDAB mole fractions ranging from 0 to 0.9 were prepared in glass vials by dissolving the lipid/surfactant mixtures in 2-3 mL of chloroform. The solvent was removed after 12 hours storage under vacuum in a desiccator, leaving the lipids cast as a thin film on the inside of the vial. This film was redispersed in 1 mL of water by repeated vortex agitation, to produce multilamellar vesicles.[11] The vesicle dispersions were probe sonicated (Soniprep 150, MSE, London, UK) for 4 min with a power output of 8-10 μ m amplitude, in order to minimise the potential effect of titanium particle debris shed from the

probe tip. Six replicate sets of vesicles were manufactured with each sample having a total lipid/surfactant concentration of 10 mg mL⁻¹.

Infra-red spectra of the vesicle suspensions were measured using a Perkin-Elmer Spectrum One FTIR Spectrometer with an FR-DTGS (fast recovery deuterated triglycine sulphate) detector. The data were processed with the corresponding Spectrum 10 Software (Version 10.4.00.0190). The samples were injected into a thoroughly cleaned liquid transmission cell (The PearlTM, Specac, Orpington, UK) with two CaF₂ windows and a defined path length of 25 µm. A spectral resolution of 4 cm⁻¹, a scan speed of 0.2 cm s⁻¹ and a strong apodization as well as a magnitude phase correction were used for each measurement. A spectral range of 3800 – 800 cm⁻¹ was scanned between 64 and 256 times depending on the signal-to-noise ratio. The background spectrum for pure water was subtracted from those of the vesicle samples, in order to achieve a flat baseline in the CH₂ stretch region. Baseline correction was also applied using the interactive baseline correction software from Spectra 10, focusing in the phosphate region with baseline points at ~1280 cm⁻¹ and ~1140 cm⁻¹. This allowed the peak heights at 1221 cm⁻¹ and 1201 cm⁻¹ from different measurements to be compared.

2.4 DSC measurements

Lipid/surfactant dispersions were prepared as described above for the zeta-potential and DLS measurements. Lipid films of different mixtures ($x_{\text{DHDAB}} = 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, \text{ and } 0.8$) and of the pure lipids were obtained by evaporating the solvent at 40°C for 1 h at 200 mbar and for a further 3 h at 10 mbar. After formation of dry lipid films, sterile filtered dispersion medium (10 mM acetate buffer pH 5, PBS, or water) was added to give a final concentration of 1.7 mM (DHDAB), 1.3 mM (DPPG) and 3 mM (lipid/surfactant mixtures), respectively. The lipid dispersions were then incubated at 60°C while shaking (1400 rpm) for 30 min. Finally, the lipid dispersions were bath sonicated at 37 kHz (100 % power output) for 6 min.

The DSC measurements of the lipid dispersions were performed on a MicroCal VP-DSC (MicroCal Inc., Northampton, MA, USA). The scanned temperature range was between 2 and 60°C, the heating rate was 60 K h⁻¹, and each heating and cooling scan was repeated up to 6 times to confirm reproducibility. In each case the results of the first scan were discarded. The reference cell was filled with pure solvent, and the buffer–buffer baseline was subtracted from the thermograms of the vesicle samples. The DSC scans were analysed using MicroCal Origin 8.0 software, with the transition temperature (T_m) assigned as the peak maximum.

3. Results and Discussion

3.1 Zeta-potential measurements and DLS

The effect of lipid ion pair formation on the surface charge density properties of mixed DPPG/DHDAB vesicles was determined by the measurement of the ζ -potential. Theoretically, with an increasing proportion of DPPG/DHDAB ion pairs (henceforth named IP(PG-DAB)) the zeta potential would decrease and reach a value of around zero if all of the lipids/surfactant molecules are associated in ion pairs. Figure 2A shows the ζ -potential as a function of the molar ratio of DHDAB in the binary lipid/surfactant mixture. At $x_{\text{DHDAB}} \leq 0.4$ the ζ -potential is negative with values below -20 mV. In this mixture, an excess of DPPG is present and consequently anionic DPPG in coexistence with IP(PG-DAB) conjugates exist and result in an excess negative charge. At $x_{\text{DHDAB}} \geq 0.6$ the ζ -potential is positive, where the excess DHDAB which is not included in ion pairs results in the positive ζ -potential. The Boltzmann fit shows that the point of charge compensation in the lipid mixture is around $x_{\text{DHDAB}} = 0.5$. This can be an indication that all of the DHDAB and DPPG molecules are intimately ion paired into tight IP(PG-DAB) clusters or, alternatively, that only a charge neutralisation occurs without influencing single lipid characteristics (as in loosely associated or separated ions).

The inflection point of the fitted zeta potential curve corresponds to the point of the lowest colloidal stability of the lipid/surfactant system (Figure 2B). This is not surprising because according to the DLVO theory, these neutral vesicles tend to agglomerate due to their low surface charge density.[13] This results in a pronounced flocculation at $x_{\text{DHDAB}} = 0.5$ and was the reason while no usable ζ -potential and FTIR measurements could be performed on samples with this 1:1 molar ratio.

3.2 FTIR measurements

The FTIR spectra of DPPG and DHDAB are shown in Figure 3. After subtraction of the water peaks and baseline correction, the following assignments were made for the DPPG spectrum which clearly shows, with good signal to noise ratios, peaks at 3000-2800 cm^{-1} (CH stretching modes), 1736 cm^{-1} (carbonyl mode), $\sim 1467 \text{ cm}^{-1}$ (CH_2 bending mode [scissoring]), $\sim 1217 \text{ cm}^{-1}$ (asymmetric PO_2 stretching mode) and the mixed band at 1130-1020 cm^{-1} (symmetric and asymmetric ester C-O stretch region, and symmetric PO_2 stretching mode). The sharp peak at $\sim 1645 \text{ cm}^{-1}$ is the remains of the water $\nu(\text{O-H})$ absorbance that was not subtracted due to the saturation of the detector in this region and therefore can be ignored. The CH_2 scissoring is typical for hexagonal chain packing [$\delta(\text{CH}_2)_n = 1468 \text{ cm}^{-1}$], and did not show any change during the measurements (single components and lipid mixtures).[14] The carbonyl band [$\nu(\text{C=O})$] was considered too close to the $\nu(\text{O-H})$ water absorbance and therefore may have been affected by the solvent subtraction. Nevertheless, no shift was observed for this band. When compared with that of DPPG, the DHDAB spectrum shows a lack of absorbance in the $\nu(\text{PO}_2)$ bands and the $\nu(\text{CO-O-C})$ bands region, therefore observed changes in the $\nu_{\text{as}}(\text{PO}_2)$ band of the binary mixtures arise only from environmental changes of the DPPG phosphate group where the influence on the absorbance from the formation of ion pairs can be observed.

The PO_2^- stretch region of DPPG was monitored as this is known to be influenced by the phosphate hydration and can therefore be used to quantify the degree of ion-pairing.[15] Sections of the $\nu_{\text{as}}(\text{PO}_2)$ stretching mode of the spectra of the liposomes composed of binary DPPG/DHDAB lipid mixtures with different molar fractions of DHDAB are shown in Figure 4. The DHDAB 0.5 mole fraction mixture did not form a stable dispersion and therefore is not shown. The data shows that the peak ratio at 1221 cm^{-1} to 1201 cm^{-1} (a_{1221}/a_{1201}) increases with increasing molar fraction of DHDAB in the DPPG/DHDAB mixtures (Figure 5).

The plot of a_{1221}/a_{1201} against DHDAB content has a sigmoidal form with the peak ratio gradually increasing until the 0.5 DHDAB mole fraction where it begins to plateau. This suggests that the change in the peak ratio is a result of the ion pairing between the head groups of DPPG and DHDAB. Above the 1:1 molar ratio it may be assumed that all of the available DPPG molecules are already involved in ion pairs, and therefore no further change to the $\nu_{\text{as}}(\text{PO}_2)$ region can be observed apart from the overall decrease in absorbance due to the dilution effect. Since DHDAB does not absorb in the $1220\text{--}1200\text{ cm}^{-1}$ region, the result indicates the phosphate of the DPPG headgroup is directly involved in the ion pairing.

The asymmetric phosphate stretch can also be regarded as an area of interest when it comes to determining changes in headgroup conformation.[16] Measurements on a DPPC/sodium cholate mixture showed a decrease in the R-O-P-O-R' shoulder at $\sim 1060\text{ cm}^{-1}$ compared to pure DPPC, but no shift for the $\nu_{\text{as}}(\text{PO}_2)$ peak, showing that the degree of hydration of the phosphate headgroup was unaffected by the presence of the bile salt.[16] Our results show a clear increase in the $\nu_{\text{as}}(\text{PO}_2)$ peak at $\sim 1221\text{ cm}^{-1}$ with increasing amounts of DHDAB, which is indicative of dehydration of the DPPG phosphate,[8, 15, 17] most likely as a direct result of ion pair formation.[18] To the best of our knowledge this is the first time changes in this region were used to assess ion pairing in long chain lipid bilayers in liposomes using FTIR.

The increase of the signal at 1221 cm^{-1} is also indicative for the onset of the formation of a DPPG/DHBD ion pair, and that this likely influences the phase transition (see DSC results below) by changing the ratios of the different attractive and repulsive forces between the molecules (Fig. 1) and thereby the packing density. Previous attempts to study hydrated liposomes with FTIR used liquid cells with path lengths of $\sim 7\text{ }\mu\text{m}$, yielded lower signal-to-noise data.[15, 19] The path length used in this study was more than 3 times higher allowing measurements with lower vesicle concentrations at better signal-to-noise ratios. Another advantage of the current approach is that the sample cell is horizontally mounted in order to reduce the loss of material from the beam focus due to sedimentation effects influencing the measurement for samples mounted in vertical liquid cells. According to the size measurements presented in Figure 2 sedimentation effects can occur at $x_{\text{DHBD}} = 0.42\text{--}0.58$. Importantly, the phosphate symmetric and asymmetric region remains accessible with the $25\text{ }\mu\text{m}$ path length allowing an accurate and reproducible subtraction of water in the background.

Aside from the inhibitive effect on the membrane permeability of cations, it has long been speculated that ion pairing also influences lipid molecular packing and bilayer order.[20] Thus a second important region of the FTIR spectrum to focus on is the CH_2 stretch region, which is also accessible with the $25\text{ }\mu\text{m}$ path length. The structural properties of the lipid/surfactant alkyl chains can be obtained from the peak position of the symmetric CH_2 stretch band at around 2850 cm^{-1} . [9, 14, 21-23] Lower values indicate a higher amount of alkyl chains in the *all-trans* conformation with a higher degree of order and tighter packing, while values above 2850 cm^{-1} indicate an increase of *gauche* rotamers representing conformational effects rather than effects on the packing geometry.[24] The peak position of symmetric CH_2 stretch mode of pure DPPG and DHBD is shown in Figure 3 with values for DPPG and DHBD at 2850.9 cm^{-1} and 2852.1 cm^{-1} respectively. Thus implying that the alkyl chains of DHBD are more disordered than those of DPPG. The $\nu_s(\text{CH}_2)$ peak position as the function of the mole

fraction of DHDAB in the binary mixtures is plotted in Figure 6. The peak position reproducibly decreases from $\sim 2850.7\text{ cm}^{-1}$ to 2850.2 cm^{-1} at around the ~ 0.5 mole fraction, where the peak position is assumed to be at its lowest value. This value is indicative of higher ordered alkyl chains with a lower amount of *gauche*-conformers which would disturb tight chain packing. When DHDAB is in excess in the membrane, the peak position shifts to higher wavenumbers in a linear trend to $\sim 2852\text{ cm}^{-1}$ at 100% DHDAB. Therefore, up until to the 0.5 mole fraction of DHDAB, the system appears to become more ordered when compared to pure DPPG even though one would expect the presence of another amphiphile would disrupt the bilayer structure. Whereas in other systems similar behaviour was assigned to H-bonding interactions[24] we attribute it in our model to ion pairing interactions in the headgroup influencing the chain order. The ion pairing also affects the interplay of intermolecular forces resulting in a tighter alkyl chain packing and higher degree of order in the alkyl chains.

3.3 Differential Scanning Calorimetry

The influence of DHDAB on the thermotropic behaviour of DPPG vesicles was investigated by DSC. The results of the experiments in pH 5 acetate buffer are presented in Figure 7. We decided to use a buffer system at pH 5 to ensure a constant pH (pH of water for FT-IR and zeta-potential measurements was between 5 and 6), sufficiently far above the pKa value of 3.5 for the phosphate of DPPG [25]. Despite the low concentration of the buffer (10 mM), we could not totally exclude ion effects on phase behaviour, therefore we carried out the appropriate controls with water as solvent for the two most prominent lipid/surfactant mixtures (Figure 8). The IR data in Figure 6 indicated that all lipid/surfactant mixtures were in the gel phase, which is corroborated by the DSC data (Figure 7A) which also demonstrates that the temperature at which the IR measurements were performed was below the observed phase transitions for all the mixtures. The thermograms for each of the DPPG/DHDAB

mixtures tested were also compared to measurements from dispersions of the pure lipid or surfactant.

The thermogram of DPPG is characterized by a pretransition at around 37°C ($L_{\beta'} \rightarrow P_{\beta'}$ transition, generally quoted at 35°C) [26] and a main transition (T_m) at 41°C ($P_{\beta'} \rightarrow L_{\alpha}$ transition, generally quoted at 41.3 - 41.5°C [26, 27], dependent on the salt concentration) [28]. The shoulder at the offset part of the transition (between 42°C and 45°C) is typical for PG bilayers in low ionic strength buffers [29]. DHDAB exhibited a phase transition with T_m at 30.5 °C (Figure 7), 2°C higher than has been previously described for DHDAB dispersions in water.[30]

Assuming ideal mixing of the components, the addition of DHDAB to DPPG should theoretically result in an continuous linear decrease of the phase transition temperature beginning at 41°C (pure DPPG) with increasing amount of DHDAB, until the phase transition of DHDAB is reached with $x_{DHDAB} = 1$ (Figure 7B, black line).[31] However, this is not observed for any of the investigated DPPG/DHDAB mixtures. From x_{DHDAB} 0.2-0.7 a pronounced stabilization of the gel phase is detectable, characterized by a shift of the phase transition to temperatures higher than the T_m of pure DPPG. This stabilized gel phase can be explained by the formation of an ion pair between DPPG and DHDAB. Theoretically and according to the FTIR and ζ -potential data presented above, the equimolar mixture ($x_{DHDAB} = 0.5$) should be characterized only by the ion pair complex formed by both amphiphiles, named IP(PG-DAB). Surprisingly, this mixture shows two phase transitions, a phenomenon associated with phase separation, which cannot be explained without further structural investigations. To evaluate whether the two phase transitions present in the $x_{DHDAB} = 0.5$ mixture were an artefact of the acetate buffer, we also performed the experiment in different solvents (Figure 8A). The two phase transitions were seen again in water, the solvent used for the IR investigations. In PBS a complex transition from 46-50°C composed of at least two

single transitions was observed, an effect of the higher amount of ions which affect the characteristic of the mixture. Ion effects of the polyanionic phosphate ions can be a possible reason for this observation. We propose, that the two transitions of the equimolar mixture (observed in water and acetate buffer, Figure 8A) result from the ion pair complex [IP(PG-DAB)]. This hypothesis is supported by the absence of peaks which can be assigned to the single lipids DPPG and DHDAB. Nevertheless, further structural investigations (e.g. x-ray or neutron scattering) at different temperatures are needed to understand the DPPG/DHDAB 1:1 mixture in detail, to investigate the structural changes which are responsible for the two phase transitions, and to examine the effects of ion pairing in the more biologically relevant fluid phase.

A complex compound like the ion pair IP(PG-DAB) seems to be responsible for the complex mixing behaviour of DPPG and DHDAB if the ion pair acts like an additional discrete amphiphile. Therefore, if we examine the mixing behaviour at x_{DHDAB} values above and below 0.5, we would in theory be examining the mixing of IP(PG-DAB) ($x_{\text{DHDAB}} = 0.5$) with either DPPG or DHDAB.

The mixing of IP(PG-DAB) with DPPG results in a further stabilization of the gel phase indicated by the T_m values of the phase transitions observed for the mixtures $x_{\text{DHDAB}} = 0.2$ - 0.4 , which are above 50°C (Figure 7A and 7B). At $x_{\text{DHDAB}} = 0.2$ a second transition with a T_m value comparable with DPPG is observed (Figure 7A and 7B) which indicates a tendency towards demixing. In the light of the DSC experiments the mixture at $x_{\text{DHDAB}} = 0.3$ has to be discussed in more detail. This mixture shows the highest phase transition temperature and no additional peak in the investigated temperature range. This means there is no phase separation (all the components are mixed) and we observe a stabilized gel phase in this mixture (azeotropic behaviour). In water there is a small additional peak in the DSC curve at temperatures higher than the main transition, and in PBS a weak transition at temperatures lower than the main transition was observed (Figure 8B). Nevertheless, the demixing effects

observed in these two media are not very pronounced. The azeotropic behaviour of the mixture between the ion pair IP(PG-DAB) and unpaired DPPG could be explained by a stabilization with hydrogen bonds. We therefore performed DSC experiments in the presence of 8M urea (Figure 8A and 8B). Urea can act as *chaotropic agent* and suppresses the formation of hydrogen bonds.[32] The effect of urea on the DSC curve of the mixture with $x_{\text{DHDAB}} = 0.5$ is negligible (Figure 8A). The curve is comparable with the experiments performed in water and acetate buffer. The effect of urea on the mixture with $x_{\text{DHDAB}} = 0.3$ is that of a more pronounced phase separation (Figure 8B) that indicates a possible role for hydrogen bonds in the intermolecular associations within this lipid/surfactant mixture. Nevertheless, the experiments did not explain the pronounced stabilization of the gel phase in these mixtures, because no shift of the phase transition to significant lower temperatures was observed in the presence of urea. This effect is more likely a result of other attractive forces such as electrostatically driven forces or Van der Waals forces. Because of the pronounced gel phase stabilization at $x_{\text{DHDAB}} = 0.3$ we propose a molecule association composed of two DPPG molecules and one DHDAB molecule which has a stabilizing effect due to the beneficial interplay of the various molecular forces illustrated in Figure 1. Due to the excess negative charge in this amphiphilic “ion triplet”, a headgroup charge delocalization could explain the reduction of lateral electrostatic repulsive forces allowing the observed bilayer stabilisation in the gel phase.

At x_{DHDAB} values above 0.5 the stabilization of the gel phase disappears. At $x_{\text{DHDAB}} = 0.6$ and $x_{\text{DHDAB}} = 0.7$ the two peaks of the equimolar mixture still appear (Figure 7B). Furthermore, both binary mixtures show a typical curve shape of a eutectic melting behaviour (Figure 7A, the marked two transition peaks which are connected for $x_{\text{DHDAB}} = 0.6$ and $x_{\text{DHDAB}} = 0.7$).[33, 34] We think this eutectic mixture is a result of the mixing of the ion pair IP(PG-DAB) with DHDAB.

4. Conclusion

The DPPG/DHDAB system is theoretically characterized by a stoichiometric charge compensation. With an equimolar lipid/surfactant mixture the ζ -potential becomes zero and the vesicles agglomerate. The FTIR and DSC experiments demonstrate that the charge compensation in the lipid system is not only a simple neutralization of charges, but rather results from the formation of ion pairs with different physico-chemical characteristics compared to pure DPPG and DHDAB. Such ion pair can be named a “cluster compound” or “complex compound” and acts as a single amphiphile with new characteristics. We found different indications for this assumption. The screening of the symmetric methylene stretching mode indicates the lowest content of *gauche* conformers, and therefore the tightest chain packing, at the charge neutral equimolar DPPG/DHDAB mixture. Also, the evaluation of the intensity ratio a_{1221}/a_{1201} of the asymmetric phosphate stretching mode indicates a plateauing of the inter-headgroup interaction and therefore the optimal ion pair formation in the equimolar mixture. The specific interaction between DPPG and DHDAB influences also the thermotropic behaviour of the binary mixture. At equimolar ratios, the ion pair formation results in a stabilization of the gel phase because the observed phase transition is higher than the transition temperatures of the pure components. Furthermore the ion pair seems to act as a cluster compound which has different mixing behaviours with excess DPPG or DHDAB. With DPPG an azeotropic mixing behaviour occurs with a further increase of the phase transition. Further experiments are required to evaluate the mechanism of this increased stabilization of the gel phase, but we favour a model of putative charge delocalization occurring when one cationic lipid interacts with two anionic lipids, in an ion triplet configuration. The interaction of the ion pair with excess DHDAB results in a eutectic mixture.

In summary, the interaction of the anionic lipid DPPG and the cationic surfactant DHDAB is not solely one of pure charge neutralization but rather results in a formation of an ion pair which acts as complex compound. The reason is the shift of the balance between different attractive and repulsive intermolecular forces driven by the electrostatic interaction between the oppositely charged amphiphiles. This has implications for the stability of Gram positive bacterial plasma membrane lipid bilayers which contain ion pairs, and may explain why relatively small quantities of cationic lipids can facilitate resistance to membrane active antimicrobials[2], by imparting enhanced physical stability rather than significant charge dampening.

Conflicts of interest

There are no conflicts to declare.

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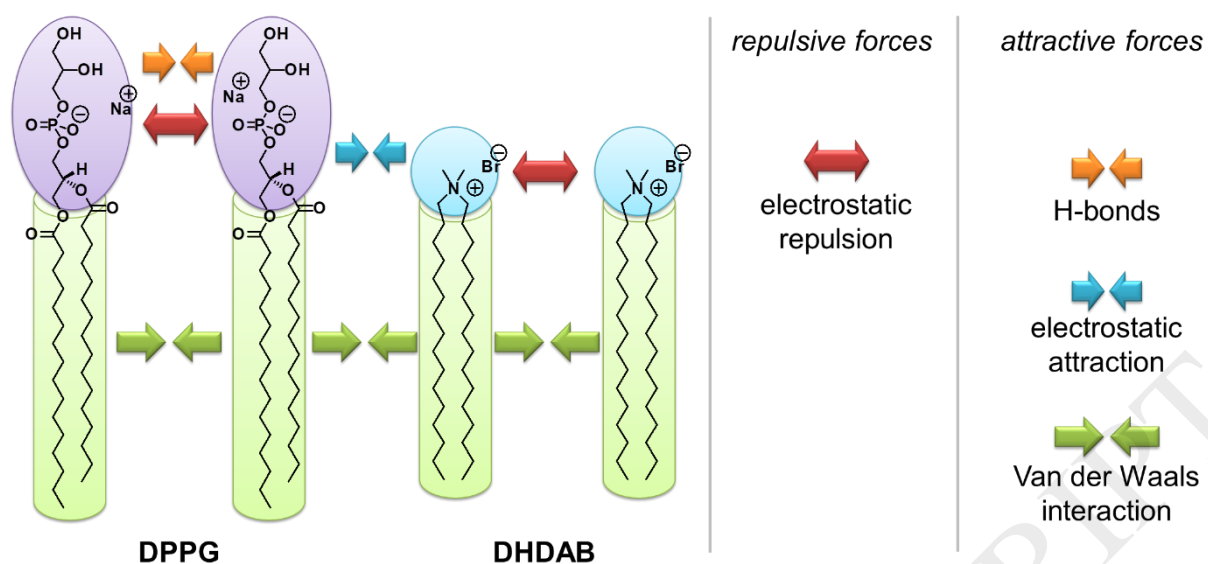


Fig. 1. Structures of the investigated lipids and schematic overview of different forces acting between the amphiphiles. DPPG = dipalmitoylglycero-3-phosphoglycerol; DHDAB = dihexadecyldimethylammonium bromide.

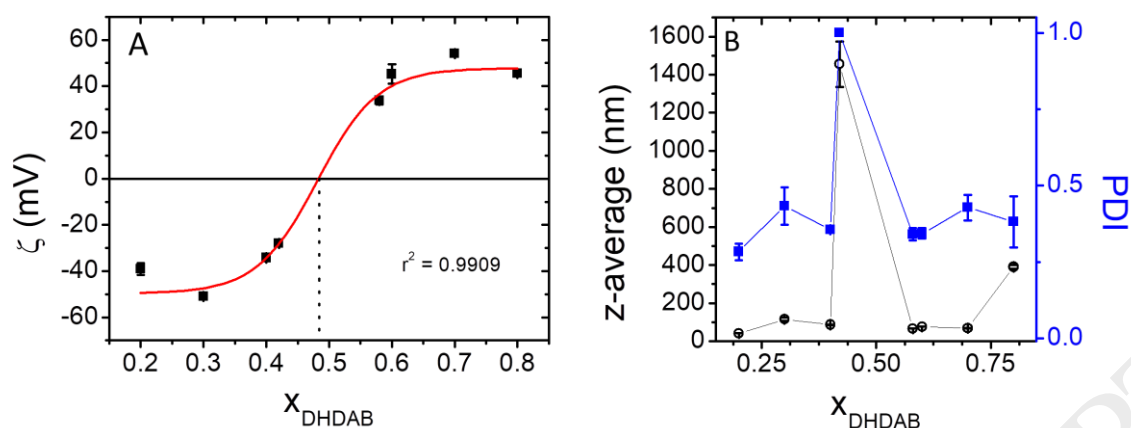


Fig. 2. A) ζ -potential of vesicles composed of DPPG/DHDAB mixtures as a function of the molar fraction of DHDAB in DPPG/DHDAB mixtures. The red curve shows the Boltzmann fit of the data. B) Combined plots from the DLS measurements. Z-average (diameter) (black circles) and polydispersity index (PDI) (blue squares) of vesicles composed of various DPPG/DHDAB mixtures as a function of the molar fraction of DHDAB in DPPG/DHDAB mixtures (data points correspond to mean \pm SD of 3 experiments).

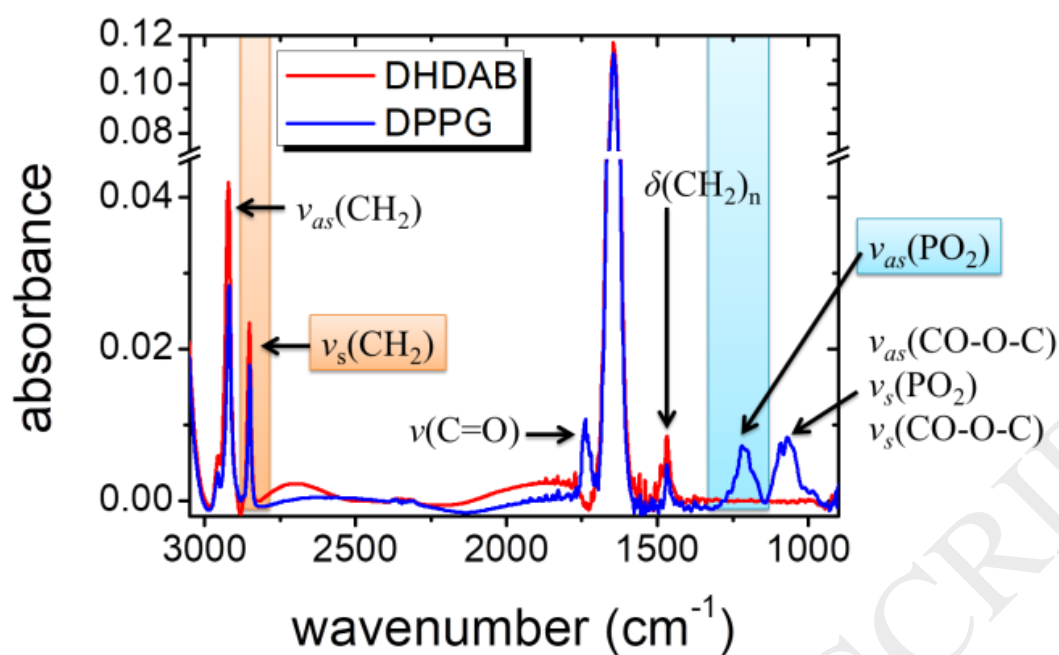


Fig. 3. FTIR spectra of DHDAB and DPPG showing typical IR modes of phospholipids/lipids.[9] The highlighted areas indicate the region of interest for the evaluation of the ion pair formation.

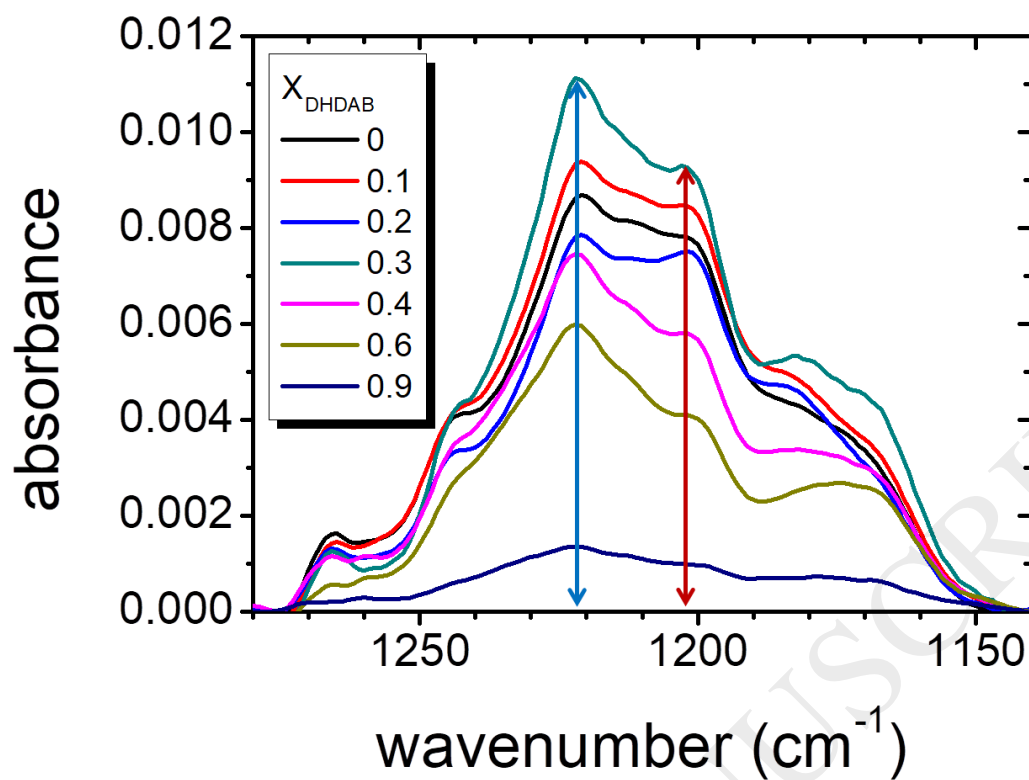


Fig. 4. The $\nu_{as}(\text{PO}_2)$ stretching mode of the spectra of different DPPG/DHDAB mixtures. The two arrows indicate the peak positions at 1221 cm^{-1} and 1201 cm^{-1} which was used to determine the a_{1221}/a_{1201} ratio.

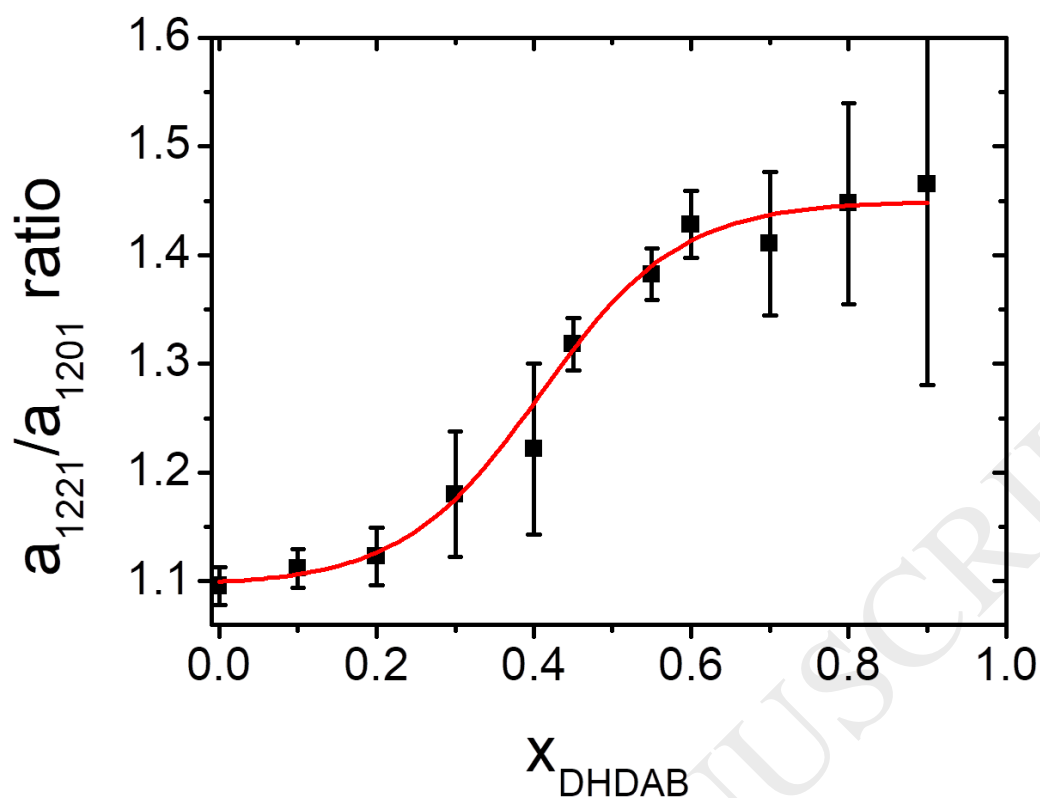


Fig. 5. The a_{1221}/a_{1201} ratio determined from the absorbance of the IR spectra at position 1221 cm^{-1} to 1201 cm^{-1} plotted as the function of the molar fraction of DHDAB in the binary DPPG/DHDAB mixtures according to Figure 4 (data points correspond to mean \pm SD of 6 experiments). Pure DHDAB has no phosphate group and was therefore not measured. The red line shows the sigmoidal fit after Boltzmann.

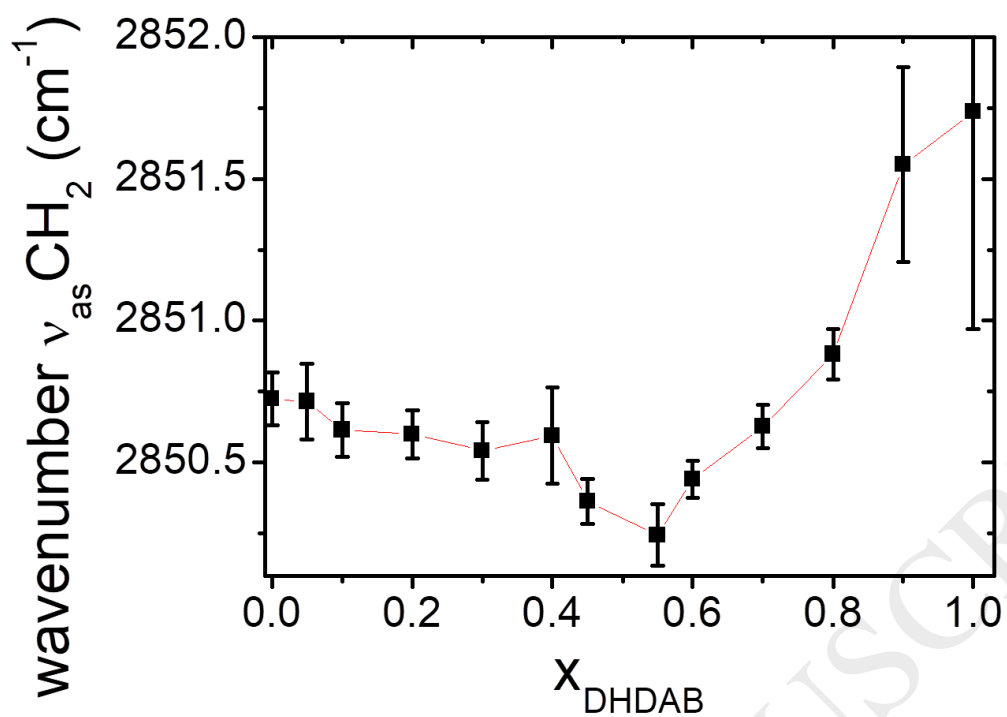


Fig. 6. The symmetric CH_2 valence vibration for DPPG and DPPG/DHDAB binary mixtures plotted against the molar fraction of DHDAB (data points correspond to mean \pm SD of 6 experiments).

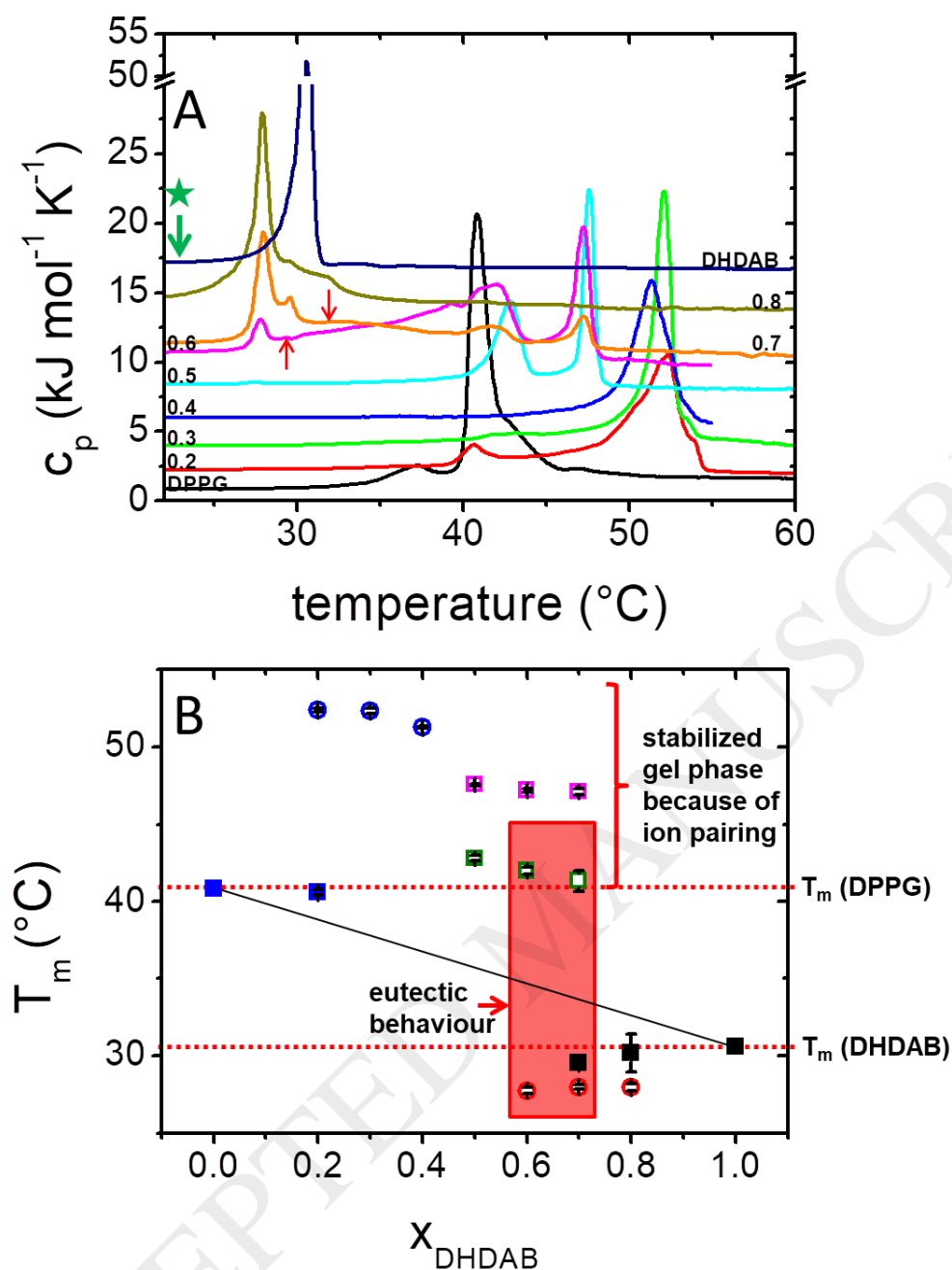


Fig. 7. A) DSC heating scans of different DPPG-DHDAB mixtures (second scan). The curves are offset vertically for clarity. The numbers displayed on each curve indicates the x_{DHDAB} values. The arrow with the star indicates the temperature at which the IR experiments were conducted. The two red arrows indicate the occurrence of typical eutectic behaviour. B) The mean T_m values of the DSC scans of the curves in A (data points correspond to mean \pm SD of 5 experiments).

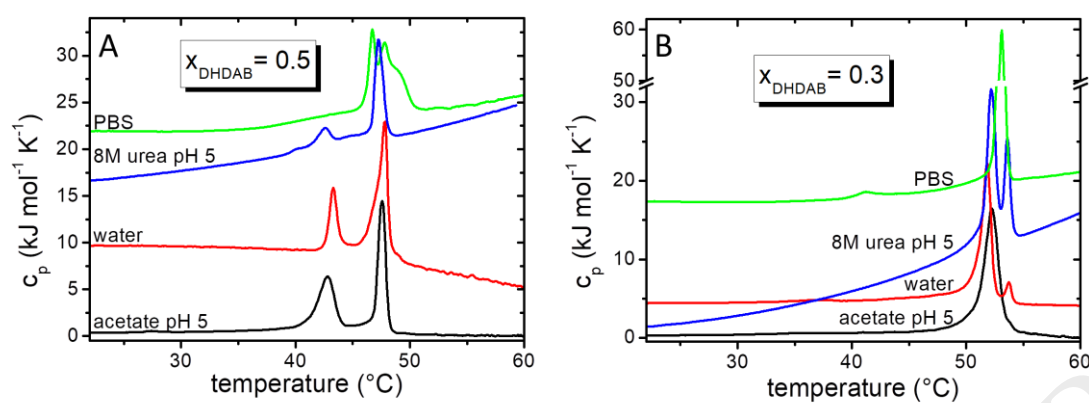


Fig. 8. DSC heating scans of two different DPPG-DHDAB mixtures in different media. The curves are offset vertically for clarity.